Selective Cytotoxicity of Purified Homologues of Tunicamycin on Transformed BALB/3T3 Fibroblasts

Miri Seiberg and Dan Duksin

Department of Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT

The selective cytotoxicity of tunicamycin homologues against SV40-transformed 3T3 cells (SV40-3T3) was examined. Incubation of 3T3 or virally transformed 3T3 cells with four different homologues (A₁, A₂, B₁, and B₂ at 0.1 to 0.25 μg/ml) caused detachment and death of transformed cells after 1 to 3 days, while the nontransformed cells were almost unaffected. Cytotoxicity against nontransformed cells occurred only when higher doses (at least 5-fold) of A₁-, A₂-, and B₂-tunicamycins were used. In contrast, these homologues inhibited proliferation of 3T3 cells, even when doses of 0.5 μg/ml were used. These cytotoxic effects are dose dependent, and maximal cytotoxicity of each homologue is achieved at a different concentration in each cell type. These results indicate that tunicamycin homologues have selective cytotoxicity against transformed cells. Incorporation of [³H]mannose into acid-precipitable macromolecules synthesized by transformed cells was strongly inhibited (70 to 75%) by A₁- and B₂-tunicamycins at 0.01 to 0.05 μg/ml, while incorporation by 3T3 cells was not affected. At higher concentrations of the above tunicamycins (0.5 to 1 μg/ml), [³H]mannose incorporation by both 3T3 and SV40-3T3 cells was inhibited more than 95%. In contrast, the effect of these tunicamycin homologues on protein synthesis in 3T3 and SV40-3T3 fibroblasts was less pronounced since the incorporation of amino acids was inhibited by approximately 20%. Very little inhibition of amino acid incorporation occurred when 3T3 or SV40-3T3 cells were treated with B₁-tunicamycin. However, A₁-tunicamycin inhibited [³H]proline incorporation and slightly increased [³H]tyrosine incorporation into cell layers of 3T3 cells. Examination of secreted proteins synthesized by these cells on sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that both 3T3 and SV40-3T3 cells treated with homologues produced partially glycosylated macromolecules, such as procollagen and fibronectin, and failed to convert procollagen to collagen. Tunicamycin homologues also inhibited the N-acetylglucosamine-1-phosphate transferase activity found in microsomes prepared from 3T3 and virally transformed 3T3 fibroblasts. The data presented indicate that the cytotoxic activity of purified homologues of tunicamycin against transformed fibroblasts might be due to the selective inhibition of glycosylation and to the differences in the membrane solubilities of the homologues.

INTRODUCTION

Tunicamycin specifically inhibits protein glycosylation by blocking the first step in the synthesis of asparagine-linked carbohydrate side chain(s) of glycoproteins, i.e., the transfer of GlcNAc-1-P³ from UDP-GlcNAc to the dolichyl monophosphate carrier (12, 29). By using tunicamycin, the role of carbohydrates in glycoprotein synthesis, processing, secretion, and biological activities has been examined in mammalian cells, viruses, yeasts, and bacteria (24). Of particular interest is the effect of tunicamycin on transformed cells. It has been reported that these cells show a higher sensitivity than normal cells to the effect of tunicamycin on transformed cells. An increase in cell morphology and adhesion, cell growth, incorporation of sugars and amino acids into acid-insoluble products, and formation of lipid-linked GlcNAc were 5 to 10 times more pronounced in tunicamycin-treated transformed cells than in normal cells (4, 6, 17, 28). Selective cytotoxicity of tunicamycin was found in UV-irradiated, virally or chemically transformed cells from different sources (23). The mechanism of the specific cytotoxicity of tunicamycin against transformed cells is not completely understood. Moreover, it is not known to date whether this cytotoxicity results from inhibition of protein glycosylation or inhibition of protein synthesis. It is now known that tunicamycin is a mixture of several homologues, which differ in their fatty acid side chains (14, 26, 27). Sixteen homologues were separated by reversed-phase high-performance liquid chromatography (20, 21), and the biological activities of the 9 major homologues (A₀-, A₁-, A₂-, B₁-, B₂-, C₁-, C₂-, D₁-, and D₂-tunicamycins) were examined using chick embryo fibroblasts (7, 8, 20). They were found to differ in the amount of antibiotic required to block protein glycosylation, in their ability to inhibit protein synthesis, and in the level of protein synthesis inhibition at concentrations that completely blocked glycosylation. Therefore, we have tested the effect of several tunicamycin homologues on cell viability and growth in culture, on protein glycosylation, on protein synthesis, and on the transfer of GlcNAc-1-P to the lipid carrier in mouse 3T3 fibroblasts and in their virally transformed counterparts. In this paper, we report that tunicamycin homologues are cytotoxic against transformed cells and inhibit protein glycosylation in such cells at lower concentrations than in 3T3 cells.

MATERIALS AND METHODS

Materials. Tunicamycin homologues were separated from tunicamycin mixture using high-performance liquid chromatography (21). Homologues were solubilized at 0.1 mg/ml in 0.01 n NaOH, lyophilized, and kept at −70°C. Samples were resolubilized in glass distilled water and kept at −20°C for not more than 2 weeks. L-[2,3-³H]Proline (29.3 Ci/mmol), L-[3,5-³H]Tyrosine (52.1 Ci/mmol), UDP-[³H]GlcNAc (6.6 Ci/mmol), and GDP-[¹⁴C]Man (192 mCi/mmol) were from New England Nuclear, Boston, Mass. ²-[²-³H]Man (12 Ci/mmol) was

¹ Supported by a grant from the Israel Academy of Sciences and Humanities. ² To whom requests for reprints should be addressed.

Received June 26, 1982; accepted November 4, 1982.
from The Radiochemical Centre, Amersham, United Kingdom. All tissue culture solutions were purchased from Bio-Lab (Jerusalem, Israel). Plastic tissue culture dishes were from Miniplast (Ein-Shemer, Israel) and Costar (Cambridge, Mass.). Dolichyl monophosphate [2 mg/ml in chloroform:methanol (2:1)] was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell Lines and Cell Culture. 3T3 and SV40-3T3 cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cultures were maintained in a humidified incubator in 95% air:5% CO₂ at 37°C.

Cytotoxicity Assay. Cells were seeded at ~10⁴ cells per 35-mm dish. After 3 days of incubation, media were replaced, and tunicamycin homologues A₁, A₂, B₁, and B₂ were added at different concentrations (0 to 0.5 μg/ml). Cell morphology was observed with a phase-contrast microscope. Growth curves were determined by counting viable cells in a hemocytometer every 24 hr. The number of viable cells were determined by repeated counting at 8-hr intervals in the presence of trypan blue (0.02%). The term "cell proliferation" is used when there was no cell death. The values are the means of counting triplicate plates in 4 different experiments.

Incorporation of Amino Acids and Sugars. Labeling of proteins synthesized by 3T3 and SV40-3T3 fibroblasts in the presence of A₁- or B₂-tunicamycin at 0 to 0.2 μg/ml was tested as described previously (5, 20). Briefly, each cell type was preincubated for 6 hr with the specific homologue, in serum-free medium (2 ml/plate) supplemented with glutamine, sodium ascorbate, and β-aminopropionitrile (5). Radiolabeled amino acid or sugar (5 μCi/ml) was added to the medium, and cultures were incubated for an additional 16 hr. Labeling was stopped by rapid cooling and by separation of cells from medium. Total incorporation was determined after trichloroacetic acid (10%) precipitation of the macromolecular fraction in the media and cell layer compartments. The values reported are the means obtained by counting duplicate samples of 4 different experiments.

Slab Sodium Dodecyl Sulfate:Polyacrylamide Gel Electrophoresis. The separation of proteins was achieved in a composite gel in which the lower 4.5 cm contained 10% acrylamide and the upper 8.5 cm contained 5% acrylamide. The buffer system used was a modification (20) of that described by Laemmli (18). All samples contained 0.1 m dithiothreitol. After electrophoresis using a commercial apparatus (Hoffer Scientific Instruments, San Francisco, Calif.), the slabs were preincubated for 20 min at 30°C prior to the addition of the labeled nucleotide. The reaction was started by addition of 0.25 μCi of UDP-[³H]GlcNAc or 0.02 μCi of GDP-[¹⁴C]Man, and samples were incubated for 5 min at 30°C. The reaction was stopped by addition of 3 ml chloroform:methanol (2:1, v/v). The organic phase was separated and washed once with 0.5 ml of 0.9% NaCl and twice with 1 ml of methanol-water (1:1, v/v). The radioactivity in the organic phase was measured in toluene after evaporation under N₂.

RESULTS

Effect of Tunicamycin Homologues on Cell Viability and Morphology. The effect of A₁-, A₂-, B₁-, and B₂-tunicamycin homologues on cell growth of 3T3 and SV40-3T3 cells is shown in Charts 1 to 4. Tunicamycin homologues were added to cultures in their log phase of growth, when cells were actively dividing. The growth of SV40-3T3 cells was inhibited even at 0.1 μg/ml, and to a larger extent with higher doses. Proliferation of 3T3 cells was not inhibited at the lower doses of homologues and only slightly inhibited at 0.25 μg/ml of each homologue. A₁-, A₂-, B₁-, and B₂-tunicamycins inhibited 3T3 cell proliferation at 0.5 μg/ml (Charts 1 to 4); some cytotoxicity toward 3T3 cells was observed when higher concentrations of A₂-, B₁-, and B₂-tunicamycins were used. A₁-Tunicamycin, at concentrations up to 1 μg/ml, inhibited 3T3 cell proliferation without exerting cytotoxicity (data not shown).

Differences were also found in the effect of the tunicamycin homologues on the morphology of 3T3 and SV40-3T3 cells. The latter, treated for 24 hr with >0.1 μg/ml of each homologue, became rounded and partially detached from the surface of the plate. Cell death occurred 2 days after the treatment. 3T3 cells, in contrast, showed morphological changes only 2 to 4 days after exposure to tunicamycin homologues at doses less than 0.5 μg/ml. These changes included cell elongation, appearance of vacuoles, and decrease in adhesion but not detachment or death.

Effect of Tunicamycin Homologues on Glycoprotein Biosynthesis. Tunicamycin homologues A₁ and B₂ exerted strong inhibition of protein glycosylation with less concurrent protein synthesis inhibition (8). Moreover, A₁-tunicamycin had no cytotoxic effect upon 3T3 cells even when high concentrations...
The ability of A₁- and B₂-tunicamycins to inhibit protein glycosylation was studied by measurements of incorporation of \[^{1}H\]mannose into glycoproteins synthesized by both cell types in culture. At high concentrations of A₁- and B₂-tunicamycins (1 and 0.5 μg/ml, respectively), these homologues inhibited completely the incorporation of \[^{1}H\]mannose into the media and cell layer compartments of both cell types (data not shown). In the transformed cells, mannose incorporation was inhibited to a high extent (75%) using lower concentrations of homologues (0.025 to 0.05 μg/ml), at which the incorporation of mannose into cell layer glycoproteins of 3T3 cells was not inhibited at all (Charts 5 and 6). Glycosylation in both 3T3 and SV40-3T3 cells was inhibited by B₂-tunicamycin to a greater extent than by A₁-tunicamycin.

The effect of A₁- and B₂-tunicamycins on the incorporation of labeled proline and tyrosine into medium and cell layer macromolecules is also shown in Charts 5 and 6 (middle and bottom). Incorporation of \[^{1}H\]tyrosine into the cell layer compartment was only slightly inhibited by B₂-tunicamycin (up to 15%) both in 3T3 and in SV40-3T3 cells. A slightly higher inhibition was observed in the media of these cells (up to 30%). However, A₁- and B₂-tunicamycin markedly inhibited (75% in cell layer at 0.01 μg/ml \[^{1}H\]proline incorporation (Charts 5 and 6). A slight increase in \[^{1}H\]tyrosine incorporation (30%) into the cell layer compartment of 3T3 cells was observed (Chart 5). The reason for these findings is not yet clear.

**Analysis of Secreted Proteins on Sodium Dodecyl Sulfate:Polyacrylamide Gel Electrophoresis.** Labeled proteins synthesized in the absence or presence of A₁-tunicamycin (0.05 μg/ml) were precipitated from culture media and analyzed by sodium dodecyl sulfate:polyacrylamide gel electrophoresis (Fig. 1). Secreted proteins labeled with \[^{1}H\]mannose showed a glycoprotein pattern typical for each cell type (Fig. 1, Slots 1 and 3). When SV40-3T3 cells were treated with A₁-tunicamycin, no mannose was incorporated into the glycopro-
Teins analyzed (Fig. 1, Slot 4). Under the same conditions, only a small reduction was found in the amount of mannose-labeled glycoproteins of 3T3 cells (Fig. 1, Slot 2), thus further demonstrating that low concentrations of A1-tunicamycin inhibited the mannosylation of secreted glycoproteins in transformed cells. Additional evidence that A1-tunicamycin inhibited protein glycosylation in the mouse lines was obtained when the pattern of secreted proteins, labeled with proline and tyrosine in the absence or presence of the homologue, was examined (Fig. 1, Slots 5 to 12). The fibronectin synthesized in the presence of A1-tunicamycin was found in the medium in decreased amounts (Fig. 1, Slots 6, 8, 10, and 12) and migrated faster, probably because it was not glycosylated (2). The conversion of procollagen to collagen was also inhibited by A1-tunicamycin, as demonstrated by the absence of collagen chains and the accumulation of procollagen (Fig. 1, Slot 6). The procollagen chains migrated faster, probably because they also were not glycosylated (Fig. 1, Slots 6 and 8). Similar results were obtained using B2-tunicamycin (data not shown).

**Effect of Tunicamycin Homologues on Glycosyltransferases in Microsome Preparations.** The inhibition of transfer of GlcNAc-1-P to the lipid carrier by A1-, B2-, and B3-tunicamycin homologues in microsome preparations from 3T3 or SV40-3T3 fibroblasts is shown in Chart 7. All the different homologues tested completely inhibited the transfer of GlcNAc-1-P. No inhibition was observed in the transfer of mannose from GDP-mannose to lipid fractions (data not shown). When the effect of different concentrations of the homologues on the transfer of GlcNAc-1-P to the lipid fraction was examined, several important observations were made. (a) The 3 homologues tested exerted different reactivities, and maximal inhibition was achieved at a different concentration for each homologue, namely 50, 20, and 5 to 10 ng/ml for A1-, B2-, and B3-tunicamycins, respectively (Chart 7). (b) Similar concentrations of tunicamycin homologues were necessary to cause the same level of inhibition of the GlcNAc-1-P transfer in 3T3 or SV40-3T3 cells (Chart 7, compare left panels with right panels). Enzymatic activity was similar in microsome preparations from 3T3 and SV40-3T3 cells also when compared on the basis of total membrane protein, i.e., 540 ± 15 (S.E.) and 500 ± 20 dpm/mg protein for microsomes from 3T3 and SV40-3T3 cells, respectively. When membranes were preincubated before addition of the labeled UDP-GlcNAc, a slightly increased activity was found in microsomes from transformed cells (705 ± 23 dpm/mg protein, compared with the incorporation of 480 ± 12 dpm/mg protein in microsomes from 3T3 cells). (c) Enzyme preparations from SV40-3T3 fibroblasts had to be preincubated with each tunicamycin homologue in order to achieve the maximal inhibition of GlcNAc-1-P transfer at any given concentration (Chart 7, right panels). In the enzyme preparations from 3T3 cells, on the other hand, the level of inhibition achieved by tunicamycin homologues was not changed after a preincubation period with the homologue. Preliminary experiments with B2-tunicamycin revealed that this preincubation period, necessary to achieve maximal inhibition of GlcNAc-1-P transfer, could be eliminated if the homologue was added together with dimethyl sulfoxide (1% final concentration), a treatment that did not change enzymatic activity.

**DISCUSSION**

Treatment of normal and transformed cells with tunicamycin caused inhibition of growth of nontransformed cells and cytotoxicity on transformed cells as well as cellular morphological...
Tunicamycin Homologues and Transformed Cells

Chart 7. The effect of A1-, B2-, and B3-tunicamycins on the transfer of GlcNAc-1-P from UDP-GlcNAc into the lipid fraction of microsome membrane preparations from 3T3 and SV40-3T3 fibroblasts. ○, homologue preincubated with microsomes for 20 min prior to addition of UDP-[14C]GlcNAc; ●, no preincubation. Incorporation of 100% is 1080 ± 30 and 1000 ± 40 dpm for 3T3 and SV40-3T3 preparations, respectively.

changes which were accompanied by changes in cell surface properties. Following our original observation (4) that tunicamycin is selectively cytotoxic against transformed human and mouse cells at 1 µg/ml, we also demonstrated that normal cells were inhibited in their growth (6). Several investigators reported similar findings (17, 23, 28). When the effect of tunicamycin was studied on preimplantation mouse embryos in tissue culture, the antibiotic caused marked changes in trophoblast adhesion to the substratum (25). Upon longer exposure of embryos to tunicamycin, a cytotoxic effect against trophoblasts was found (1, 30). This was attributed to the “transformed” phenotypic behavior of trophoblasts, which resemble transformed cells in their migratory properties, invasiveness, and secretion of plasminogen activator (1). Recently, tunicamycin was found to be cytotoxic against several human lymphoblastoid B-cell lines, e.g., Raji and RPMI 4098, while other lines, e.g., Wil-2, were less sensitive (10). This differential cytotoxicity was used recently in order to study metastatic properties of murine melanoma B16 cells in vivo; tunicamycin caused morphological changes, cell rounding, inhibition of growth, and reduced lung colonization potential (13). However, all these studies were performed with crude preparations of tunicamycin, composed of a mixture of homologues (14) which differ in their relative amounts (7) and in their biological activities (8, 9, 15, 20). Some of the purified homologues inhibited protein synthesis, and this could account for the cytotoxicity.

The experiments presented here demonstrated that purified homologues of tunicamycin are at least 5 times more cytotoxic against transformed 3T3 cells, and in lower doses they selec-
(14), in their cytotoxic activity, and in their ability to inhibit protein glycosylation and synthesis. The selective effect of tunicamycin homologues against transformed cells may be due to differences in membrane composition of cells, in the glycosyltransferase enzymes affected by the drug, or in the penetration of the antibiotic into the rough endoplasmic reticulum.

It is of interest to determine whether tunicamycin homologues with high selectivity in vitro will exert specific cytotoxicity on tumors in vivo.

ACKNOWLEDGMENTS

We thank Dr. W. C. Mahoney for fruitful discussions.

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